VERIFICATION OF TRANSLATION

I, <u>Ryoko OSHIKAMO, registered Patent Attorney , having my business place at Fukuoka Building, 9th Floor 8-7, Yaesu 2-Chome, Chuo-ku, Tokyo 104-0028 Japan,</u> state the following:

I am fluent in both the English and Japanese languages and capable of translating documents from one into the other of these languages.

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I state that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

Signature : _Ryoko Oshikamo_

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Date : _February 15, 2006_

PROMOTER OF SYNOVIOLIN GENE

5   <u>TECHNICAL FIELD</u>

The present invention relates to a promoter of the synoviolin gene.

<u>BACKGROUND ART</u>

Synoviolin is a protein that was cloned from synoviocytes library of patients with
10   rheumatoid arthritis by immunoscreening using anti-synovial cell antibodies.

Synoviolin is a ubiquitin ligase (E3) having a ring finger domain, and has a quality
control function in the endoplasmic reticulum (ER). The amino acid sequence (nucleotide
sequence) of synoviolin (the synoviolin gene) is highly homologous with yeast Hrd1, and has
been shown to be the same molecule as human hrd1, which was cloned as a human homolog
15   of yeast Hrd1 (Kaneko, FEBS Lett., Dec. 4, 532 (1-2): 147-52, 2002).

The following three mechanisms for quality control in the endoplasmic reticulum have
already been discovered. One mechanism involves PERK (endoplasmic reticulum resident
kinase). This mechanism regulates translation and reduces ER stress (explained below) by
phosphorylating the translation initiation factor elf. The second is a mechanism of translation
20   regulation via the ATF6 and XBP1 transcriptional factors by means of ATF6, IRE-1 and other
ER transmembrane proteins that sense ER stress. In this mechanism, ATF6 and XBP1 induce
transcription of Bip and other chaperone molecules, promoting a re-folding reaction of
defective proteins. This reaction is called the UPR (unfolded protein response). The third is
ERAD (ER-associated degradation), which reduces ER stress by breaking down proteins
25   (Kaufman, RJ, Nat. Rev. Mol. Cell Biol., June, 3(6): 411-21, 2002). ERAD can be explained
in detail as follows.

A protein which has been synthesized in the cytoplasm can only function when it has
formed its proper three-dimensional structure and been transported to its assigned place. An
unfolded or damaged protein which has not assumed an appropriate higher structure is
30   checked by the quality control function of the cell, and is regenerated or broken down to
maintain normal cell function.

Because proteins are unstable during the course of biosynthesis inside the endoplasmic
reticulum lumen, they are exposed to various kinds of physical and chemical stress (including

ischemia, hypoxia, heat shock, amino acid starvation, genetic mutation and the like). This kind of stress, called endoplasmic reticulum stress (ER stress), increases the occurrence of proteins having abnormal folding structures in the endoplasmic reticulum (unfolded proteins). Because defective proteins with three-dimensional structure abnormalities are not transported from the endoplasmic reticulum to the Golgi apparatus, unfolded proteins accumulate in the endoplasmic reticulum. In response to such ER stress, therefore, the cell breaks down the defective proteins by means of the endoplasmic reticulum-specific stress response mechanisms called UPR and ERAD, thus preventing stress to the ER from accumulation of such defective proteins. Synoviolin is induced by ER stress and is involved in the third mechanism, ERAD (Kaneko, FEBS Lett., Dec. 4, 532 (1-2): 147-52, 2002).

The inventors have also shown that increased expression of synoviolin suppresses sensitivity to apoptosis due to ER stress by promoting ERAD, and apoptosis sensitivity increases when synoviolin expression is lowered.

It has also been confirmed that expression is greater in rheumatic synovial cells than in normal synovial membrane. To verify the significance of synoviolin in arthritis, CIA (collagen induced arthritis: a mouse model in which bovine type II collagen is injected subcutaneously to immunize mice and cause arthritis by means of the resulting antibodies) was induced in a synoviolin hetero-knockout mouse. As a result, sensitivity to apoptosis due to ER stress was lower than in wild type mice, indicating CIA resistance. This seems to indicate that the requirements for expression are cell- and tissue-specific.

Based on these findings, since the mechanisms which regulate synoviolin levels control sensitivity to apoptosis in response to ER stress in cells, the elucidation of the mechanism is quite important.

DISCLOSURE OF THE INVENTION

It is an object of the present invention to provide a promoter of the synoviolin gene.

The inventors completed the present invention as a result of exhaustive research aimed at resolving the aforementioned problems when they cleaved the synoviolin promoter region and, after investigating promoter activity in fragments of various lengths, succeeded in identifying the core region and elucidating its interactions with transcriptional factors.

That is, the present invention is as follows.

(1)   A promoter of the synoviolin gene, comprising at least the nucleotide sequence of nucleotides 2120-2130 in the nucleotide sequence represented by SEQ ID: NO 1 or 2.

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(2)   A promoter of the synoviolin gene, comprising any nucleotide sequence selected from a group consisting of at least the nucleotide sequences of the regions of nucleotides 1-2201, 969-2201, 1142-2201, 1699-2201, 1880-2201, 2002-2201, 2094-2201 and 2118-2201 in the nucleotide sequence represented by SEQ ID: NO 1 or 2.

(3)   A promoter of the synoviolin gene, comprising any nucleotide sequence selected from a group consisting of at least the nucleotide sequences of the regions of nucleotides No. 1-3043, 969-3043, 1142-3043, 1699-3043, 1880-3043, 2002-3043, 2094-3043 and 2118-3043 in the nucleotide sequence represented by SEQ ID: NO 1.

(4)   A promoter of the synoviolin gene, comprising any nucleotide sequence selected from a group consisting of at least the nucleotide sequences of the regions of nucleotides No. 1-3092, 969-3092, 1142-3092, 1699-3092, 1880-3092, 2002-3092, 2094-3092 and 2118-3092 in the nucleotide sequence represented by SEQ ID: NO 2.

(5)   The promoter of (a) or (b) below:

(a)   A promoter of the synoviolin gene, comprising a nucleotide sequence having a deletion, substitution or insertion in some region of the nucleotide sequence of the promoter according to any one of (1) to (4) above, and having promoter activity;

(b)   A promoter of the synoviolin gene, comprising a nucleotide sequence which hybridizes under stringent conditions with a sequence complementary to the nucleotide sequence of the promoter according to any one of (1) to (4) above, and having promoter activity.

(6)   A gene expression cassette comprising the promoter according to any one of (1) to (5) above, a target gene for expression and a terminator.

(7)   A recombinant vector comprising the gene expression cassette according to (6) above.

(8)   A transformant comprising the recombinant vector according to (7) above.

(9)   A method for regulating transcription activity wherein the activity of the promoter according to any one of (1) to (5) above is inhibited or enhanced.

Inhibiting or enhancing the aforementioned promoter activity means inhibiting or enhancing the binding activity of a transcriptional factor. An example of a transcriptional factor binding site is the Ets binding site. Examples of transcriptional factors include any selected from the group consisting of GABPα, GABPβ, a complex of GABPα and GABPβ, Ets1, Pea3, Tel and Fli-1 for example.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a shows the activity of cleaved synoviolin promoters.

Figure 1b shows the activity of cleaved synoviolin promoters.

Figure 1c shows the Ets binding site and core region of a synoviolin promoter.

Figure 1d shows transcription activity with a mutation introduced into the promoter.

Figure 2a is a photograph showing the results of gel shift assay.

Figure 2b is a photograph showing the results of gel shift assay.

Figure 2c is a photograph showing the results of gel shift assay.

Figure 2d is a photograph showing the results of gel shift assay.

Figure 3a shows the transcription activity of a synoviolin promoter.

Figure 3b shows the transcription activity of a synoviolin promoter.

Figure 3c shows the transcription activity of a synoviolin promoter in NIH3T3 cells knocked out using RNAi.

Figure 4a is a structural diagram of plasmids having LacZ bound to 3k and 1k synoviolin promoters.

Figure 4b is a photograph showing expression of a synoviolin promoter in a mouse embryo.

Figure 4c is a photograph showing expression of a synoviolin promoter in a mouse embryo.

Figure 5 is a photograph showing expression of a synoviolin promoter in RA synovial membrane cells.

Figure 6a shows photographs of apoptosis in NIH3T3 cells in which synoviolin has been knocked down using RNAi, along with the results of western blotting.

Figure 6b shows photographs of apoptosis in NIH3T3 cells in which synoviolin has been knocked down using a decoy ODN, along with the results of western blotting.

Figure 7a is a photograph showing cells in which synoviolin is overexpressed.

Figure 7b is a photograph showing suppression of synoviolin expression by EBS decoy ODNs.

Figure 7c is a photograph showing apoptosis caused by EBS decoy ODNs treatment in NIH3T3 cells in which synoviolin is overexpressed.

Figure 7d shows apoptosis caused by EBS decoy ODNs treatment in NIH3T3 cells in which synoviolin is overexpressed.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention is explained in detail below.

1.   Outline

The inventors are presently analyzing pathogenesis of RA with a focus on synoviolin. We recently discovered from an analysis using a synoviolin knockout mouse that synoviolin levels which functions in ERAD are involved in threshold values of apoptosis induction in response to ER stress.

We therefore performed a promoter analysis to elucidate the transcriptional mechanisms regulating synoviolin levels, which determines the sensitivity of apoptosis induction.

At present, one mechanism responsible for inductive synoviolin gene expression is thought to be transcriptional regulation by means of XBP-1 spliced via IRE-1 which has been phosphorylated after sensing ER stress. This regulation mechanism is as follows. When unfolded proteins accumulate in the ER, the ER-localized membrane protein IRE-1 dimerizes and undergoes intermolecular self-phosphorylation. Endonuclease-like domains within IRE-1 are activated as a result and splice immature XBP-1 mRNA, producing mature XBP-1. XBP-1 is thought to promote transcription of ER molecule chaperone genes and the like.

However, there have been no reports on constitutive gene expression. We therefore began by using a mouse cell strain and mouse embryo to determine the site responsible for constitutive gene expression.

As a result, EBS was identified as being responsible for constitutive gene expression, and its element was shown to be essential for synoviolin expression in vivo. In NIH3T3 cells, it was shown that a member of the Ets family, the GABP α/β complex, is involved in transcriptional regulation via EBS.

Hetero-knockout (LacZ knock in mouse) expression analysis and northern and western analysis have shown that synoviolin is expressed ubiquitously. That is, it appears that synoviolin expression is necessary in many cells. This was deduced from the fact that the synoviolin knockout mouse died in embryo due to systemic apoptosis promotion. Expression may be stronger or weaker, however, and expression is particularly strong in cells with strong secretory ability (pancreas, testes, and nerve cells). This suggests that especially high synoviolin levels expression are required in some cells.

To understand the transcriptional regulation that determines synoviolin levels, we performed a promoter analysis in the present invention. Mechanisms of transcriptional

regulation include (i) constitutive gene expression including basic transcriptional complexes and (ii) induced gene expression in response to a stimulus. It has been reported in the past that synoviolin is induced in response to ER stress, but there have been no reports on constitutive gene expression. The inventors therefore investigated the elements responsible for constitutive gene expression.

No TATA box or initiator sequence is present in the synoviolin promoter. In the case of such promoter structures, transcriptional factors of the SP1 and Ets families and the like are important for inducing transcription.

The Ets family is a transcriptional factors having a domain called Ets which has been conserved from yeasts to humans. This family has more than 30 members, most of which are involved in transcriptional activation of molecules responsible for differentiation, proliferation and apoptosis (D.K. Watson and A. Seth, Oncogene review issue, Dec. 18, 19(55), 2000).

Of this Ets family, the transcriptional factor called GABPα has certain features related to transcriptional regulation of target genes. First, GABPα has DNA binding ability due to its Ets domain but no transcriptional activation ability. By contrast, the GABPα cofactor GABPβ has no DNA binding ability but induces transcriptional activity when it forms a dimer with GABPα, and acquires even higher transcriptional activation ability when it forms a hetero-tetramer (Yu, M., J. Biol. Chem. Nov. 14, 272(46), 29060-7, 1997). Moreover, GABPα can function as an initiator in expression of genes having no TATA box or genes having multiple transcription initiation sites (Yu, M., J. Biol. Chem. Nov. 14, 272(46): 29060-7, 1997; Kamura, T., J. Biol. Chem. Apr. 25; 272(17): 11361-8, 1997). Although GABPα is expressed ubiquitously, it acts synergistically with partner transcriptional factors binding to other sites to control expression of genes involved in cell-specific differentiation and proliferation (Schaeffer, L, EMBO J. Jun. 1; 17(11): 3078-90, 1998).

The inventors therefore determined the elements responsible for constitutive gene expression of synoviolin and investigated the significance of those elements in NIH3T3 cells.

2.   Promoter

A promoter region can be obtained by cleavage using a restriction enzyme from the synoviolin gene or a mouse or human genome sequence or the like. In general, however, there may not be a convenient restriction enzyme site located at a suitable position. The desired promoter region can therefore be obtained by PCR amplification using primers that

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already have restriction enzyme recognition sites. A desired promoter region can also be chemically synthesized based on the nucleotide sequence data for a known promoter region.

(1)   Promoter sequence

The total promoter sequence of the mouse synoviolin gene is shown by SEQ ID: NO 1, and the total promoter sequence of the human synoviolin gene by SEQ ID: NO 2, but the promoter sequence is not limited to these as long as it has actual transcription activity, the full length promoter can be cleaved and the nucleotide sequence can also be mutated by a deletion, insertion, substitution, addition or the like in part of its sequence. However, because in the present invention introduction of a mutation into the core region (comprising the Ets binding site) is likely to cause a diminution in promoter activity, mutations into the core region are preferably introduced when the aim is to suppress promoter activity.

Normal site-directed mutagenesis methods can be adopted for introducing gene mutations, and a Gene Tailor™ Site-Directed Mutagenesis System (Invitrogen), TaKaRa Site-Directed Mutagenesis System (Mutan-K, Mutan-Super Express Km: Takara Bio) or the like can be used for example.

In the promoter of the present invention, the core region which is central to its function is the region of the nucleotide sequence shown by SEQ ID: NO 1 or 2 from nucleotides 71 (−71) to 81 (−81) moving upstream (in the 5' direction) from the transcription initiation site (t of position 2201 in the nucleotide sequence shown by SEQ ID: NO 1 or 2) as the starting point, or in other words the region of nucleotides 2120 to 2130 in the nucleotide sequence shown by SEQ ID: NO 1 or 2.

Consequently, the length is not restricted as long as the aforementioned core region is included of the total sequence shown by SEQ ID: NO 1 or 2. For example, the full-length and cleaved sequences shown in Tables 1 and 2 below (see example 1), which include the aforementioned core sequence, are included in the promoter of the present invention. Moreover, a sequence which hybridizes under stringent condition with a sequence complementary to a sequence comprising the aforementioned core sequence in the promoter sequence represented by SEQ ID: NO 1 or 2 or a cleaved promoter sequence thereof, and which has synoviolin promoter activity, is included in the promoter of the present invention.

In the present invention, when the location of a promoter region is indicated it may be specified either based on the nucleotide numbers of SEQ ID: NO 1 or 2 or based on the translation initiation site. When the translation initiation site is specified, the translation initiation site (TS) is the thymidine (t) of position 2201 in the nucleotide sequence of SEQ ID:

NO 1 or 2. Starting at the TS, upstream (5') positions are indicated with negative (−) numbers and downstream (3') positions with positive numbers (+). In the 5' direction, the nucleotide 1 nucleotide upstream (5' direction) from the TS is designated −1, while in the 3' direction the TS nucleotide is designated +1. For example, the first guanine (g) of the nucleotide sequence represented by SEQ ID: NO 1 is in the −2201 position starting from the TS, while the 3043rd guanine (g) in the nucleotide sequence represented by SEQ ID: NO 1 is in the +843 position starting from the TS.

"Stringent conditions" mean conditions in which the salt concentration for washing during hybridization is 100 to 500 mM or preferably 150 to 300 mM and the temperature is 50 to 70°C or preferably 55 to 65°C. "Promoter activity" means that transcription results when a transcriptional factor binds to the promoter region of the present invention.

DNA fragments which "hybridize under stringent conditions" include those which may hybridize under the aforementioned stringent conditions even if there is a deletion, replacement, insertion or addition to some region of the nucleotide sequence. Because the promoter occurs in cleaved forms of a variety of lengths there is no particular limit on the number of the aforementioned deletions, substitutions, insertions, additions and the like as long as promoter activity is retained, but an example would be a fragment having the aforementioned mutations in one or multiple (1 to about 10 or preferably 1 to about 5) nucleotides.

(2)   Construction of recombinant vector

The recombinant vector of the present invention can be obtained by inserting the promoter of the present invention, the target gene for expression and a terminator into an appropriate vector. This is called a "gene expression cassette". Examples of vectors used for gene expression cassettes include pBR, pUC, pBluescript and other *E. coli* plasmid vectors, pcDNA3 mammal cell expression plasmid vectors and adenoviruses, retroviruses and other virus vectors. The target gene for expression need not be the synoviolin gene, and may also be a heterologous gene (such as IL-1, IL-6, TNFα and other cytokine genes, enzyme genes, growth factor genes, chemokine genes and the like). A person skilled in the art can easily insert the constituent elements of the expression vector into the vector using well known techniques. Selection marker genes and the like can also be included in the aforementioned vector. Examples of selection marker genes include G-418 and other antibiotic resistance genes, and the thymidine kinase gene, diphtheria toxin gene and the like.

There are no particular limits on the terminators that can be used in the present

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invention, but examples include the poly A signal and the like.

(3) . Transformant

Common techniques used in transforming to hosts can be adopted for introducing the plasmid into the host. That is, the protoplast method, lithium method, electroporation method and modifications of these that are well known to those skilled in the art can be applied. There are no particular limitations on the host, and examples include *E. coli* and other bacteria, yeasts, animal cells, insect cells and the like.

3. Functional analysis of the promoter

(1) Analysis of transcription activity

The transcription activity of the promoter can be analyzed by ordinary luciferase assay, gel shift assay, CAT assay or the like. Kits for performing these assays are commercially available (such as the Promega dual luciferase assay kit).

In the case of a luciferase assay for example, the firefly luciferase gene is attached as a reporter upstream from the transcription initiation site of the target gene. A vector having the cytomegalovirus (CMV) beta-galactosidase (β-gal) gene attached downstream from the promoter as a reporter can be introduced into the cell at the same time to correct the efficiency of introduction between cells which are the target of the assay. The calcium phosphate method for example can be adopted for introduction into cells. Cells with the introduced vector are collected after a fixed culture time and disrupted by freezing-liquefaction or the like, and luciferase and β-galactosidase activity are measured using a fixed amount of cell extract.

(2) EMSA

Cell differentiation and activation is controlled by gene expression, and regulation of gene expression on the transcriptional level plays a particularly important role. It is becoming clear that most transcriptional factors bind in different ways to DNA response elements. One method of analyzing the complex relationships between these transcriptional factors is gel shift assay (EMSA: electrophoretic mobility shift assay). Gel shift assay is a method in which a radioactively or non-radioactively labeled probe DNA fragment is mixed with a DNA-binding protein, and electrophoresed with a non-denatured gel with a low salt concentration, and the DNA is detected by autoradiography or the like. Because DNA binding to the protein moves more slowly through the gel than free DNA, it can be detected as a specific band. When specific antibodies for the DNA-protein complex are applied, the rate of movement

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falls still further (called a supershift). Many transcriptional factors can be identified by this method.

In cells, however, some of the secretory proteins, membrane proteins and other proteins synthesized by way of the endoplasmic reticulum acquire defects in the endoplasmic reticulum, and breakdown of these defective proteins requires expression of molecules responsible for ERAD, which is an essential mechanism in the cell. This is clear from the fact that synoviolin knockout mice die in embryo, as shown in the examples below. That is, a minimum level of synoviolin expression is required by cells. The necessary site for this transcriptional regulation is the EBS (Ets binding site). Abnormalities in signaling via this EBS site act negatively on the maintenance of cell function. On the other hand, the members of the Ets family are diverse and expressed in diverse ways, and bind to the site synergistically with other partners. This means that the transcriptional regulation mechanisms of various members of the Ets family are involved in the EBS site.

Therefore, by promoting or inhibiting the action of a protein that binds to the EBS site it is possible to regulate the transcriptional activity function of the promoter of the present invention.

Examples of transcriptional factors include GABPα, GABPβ, a complex of GABPα and GABPβ, Ets1, Pea3, Tel and Fli-1 for example.

GABPα, a member of the Ets family having an Ets domain, is a protein consisting of 454 amino acids. GABPα is expressed ubiquitously in cells. It forms a hetero-dimer with GABPβ via the Ets domain. It is also known to form a hetero-tetramer using two Ets binding sites.

GABPβ is a cofactor of GABPα having 382 amino acids. It is not a member of the Ets family. It forms a hetero-dimer with GABPα via the Ankyrin repeat sequence. It has a transcription activity domain.

Ets1 is a protein having 441 amino acids, and is a human homolog of v-ets, which was discovered in 1983 as a proto-oncogene of the retrovirus E26, which causes erythroblastosis in chickens.

Tel is a protein having 452 amino acids, and has been reported to be a member of the Ets family which functions in suppressing transcription. Clinically, it is known to cause leukemia by forming a fused protein with AML1 by means of chromosomal translocation of t (12;21).

Fli-1 is known to cause Ewing's tumor by forming a fused protein with EWS by

means of chromosomal translocation of t (11;22), and it has 452 amino acids.

The present invention is explained in more detail below using examples. However, the present invention is not limited by these examples.

5   [Example 1]   Preparation of synoviolin promoter

(1)   Plasmid construction

A roughly 7.5 kbp genome from the 5' to the 3' end comprising the synoviolin gene was subcloned from a mouse (C57B1/6) genome into SyB/pBluescript. Next, the promoter region of the synoviolin gene was treated with XhoI and NcoI, and a roughly 3k fragment was

10   removed and inserted (SyG-2.2k) into PGV-B2 (TOYO INK GROUP). This roughly 3k fragment was the full-length promoter (SEQ ID: NO 1). Certain regions were also deleted from the 5' end of the full-length promoter to prepare constructs with abbreviated promoter regions. The resulting promoters can be summarized as follows.

15   Table 1   Table of Prepared Promoters (Mouse)

Name	Region*	Region of nucleotide sequence shown by SEQ ID: NO 1
−2201/+843 (full-length)	Region of 2201 nucleotides upstream (5') and 843 nucleotides downstream (3') starting at TS (translation initiation site: +1)	1-3043
−1233/+843	Region of 1233 nucleotides upstream and 843 nucleotides downstream starting at TS	969-3043
−1060/+843	Region of 1060 nucleotides upstream and 843 nucleotides downstream starting at TS	1142-3043
−503/+843	Region of 503 nucleotides upstream and 843 nucleotides downstream starting at TS	1699-3043
−322/+843	Region of 322 nucleotides upstream and 843 nucleotides downstream starting at TS	1880-3043
−200/+843	Region of 200 nucleotides upstream and 843 nucleotides downstream starting at TS	2002-3043
−108/+843	Region of 108 nucleotides upstream and 843 nucleotides downstream starting at TS	2094-3043
−84/+843	Region of 84 nucleotides upstream and 843 nucleotides downstream starting at TS	2118-3043
−73/+843	Region of 73 nucleotides upstream and 843 nucleotides downstream starting at TS	2129-3043
−65/+843	Region of 65 nucleotides upstream and 843 nucleotides downstream starting at TS	2137-3043
−39/+843	Region of 39 nucleotides upstream and 843 nucleotides downstream starting at TS	2163-3043
−10/+843	Region of 10 nucleotides upstream and 843	2191-3043

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	nucleotides downstream starting at TS	

*The first nucleotide on the 5' side of the TS (t #2201 in SEQ ID: NO 1) is counted as −1 moving upstream (toward 5'), while the TS is counted as +1 moving upstream (toward 3').

Cleaved promoters of the human synoviolin gene can be prepared in the same way as for mice. The locations of the cleavage sites in this case are shown in Table 2.

Table 2   Table of Prepared Promoters (Human)

Name	Region*	Region of nucleotide sequence shown by SEQ ID: NO 2
−2201/+892 (full-length)	Region of 2201 nucleotides upstream (5') and 892 nucleotides downstream (3') starting at the TS (translation initiation site: +1)	1-3092
−1233/+892	Region of 1233 nucleotides upstream and 892 nucleotides downstream starting at TS	969-3092
−1060/+892	Region of 1060 nucleotides upstream and 892 nucleotides downstream starting at TS	1142-3092
−503/+892	Region of 503 nucleotides upstream and 892 nucleotides downstream starting at TS	1699-3092
−322/+892	Region of 322 nucleotides upstream and 892 nucleotides downstream starting at TS	1880-3092
−200/+892	Region of 200 nucleotides upstream and 892 nucleotides downstream starting at TS	2002-3092
−108/+892	Region of 108 nucleotides upstream and 892 nucleotides downstream starting at TS	2094-3092
−84/+892	Region of 84 nucleotides upstream and 892 nucleotides downstream starting at TS	2118-3092
−73/+892	Region of 73 nucleotides upstream and 892 nucleotides downstream starting at TS	2129-3092
−65/+892	Region of 65 nucleotides upstream and 892 nucleotides downstream starting at TS	2137-3092
−39/+892	Region of 39 nucleotides upstream and 892 nucleotides downstream starting at TS	2163-3092
−10/+892	Region of 10 nucleotides upstream and 892 nucleotides downstream starting at TS	2191-3092

*The first nucleotide on the 5' side of the TS (t #2201 in SEQ ID: NO 1) is counted as −1 moving upstream (toward 5'), while the TS is counted as +1 moving upstream (toward 3').

Next, SyG-2.2kG-76T/BV2 was prepared using site-directed mutagenesis by overlap extension (Molecular cloning, CSHL Press, 3rd Edition, 2001, Chapter 13) for purposes of preparing mutants of the aforementioned mouse-derived promoters. The following primers were used.

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1. EBSm (G-76T): GCGCCGCCGTAAGTGAGGT (SEQ ID: NO 3)

2. AMLm (G-68T): AAGTGAGTTGTCTTACCCCC (SEQ ID: NO 4)

3. SP1m (G-92A, C-91A): ACTCCGCCAAGCCCCGCGCC (SEQ ID: NO 5)

PCR was performed under the following conditions using a reaction composition comprising 1 pmol SyB/pBluescript, 100 pmol primer, 0.2 mM dNTPs, 5U polymerase, 10 mM Tris-HCl (pH 8.3) and 50 mM KCl in 50 µl of reaction solution.

PCR conditions:

First stage: 94°C, 1 minute → (94°C, 30 sec → 55°C, 30 sec → 72°C, 1 min) x 25 times

Second stage: 1st cycle: 94°C, 1 min → 55°C, 30 sec → lowered to 30°C over 29 minutes → 30°C, 1 min → to 72°C over 9 minutes → 72°C, 1 min → (94°C, 30 sec → 55°C, 30 sec → 72°C, 1 min) x 25 times

EBSm (G-76T) is a primer for mutating G to T in the 2125th position (76th (−76) upstream from the TS) in the nucleotide sequence shown by SEQ ID: NO 1, AMLm (G-68T) is a primer for mutating G to T in the 2133rd position (68th (−68) upstream from the TS) of the nucleotide sequence shown by SEQ ID: NO 1, SP1m (G-92A) is a primer for mutating G to A in the 2111th position (92nd (−92) upstream from the TS) in the nucleotide sequence shown by SEQ ID: NO 1, and SP1m (G-92A, C-91A) is a primer for mutating C to A in the 2112nd position (−91 from TS) in the nucleotide sequence shown by SEQ ID: NO 1 and mutating G to A in the 2111st position (−92 from TS) of the nucleotide sequence shown by SEQ ID: NO 1).

[Example 2] Functional analysis of synoviolin promoter

A promoter analysis was performed to elucidate the mechanisms regulating synoviolin levels. Synoviolin has been shown from transgenic mouse (LacZ knock in) analysis and northern and western results to be expressed ubiquitously.

After the synoviolin promoter had been cloned, a region comprising 2.2k from the translation initiation site was bound to a luciferase vector. The transcription activity of various constructions cut from the upstream side was investigated using a variety of cells (shown below). The cells were cultured using DMEM medium (Life Technologies, Inc.) with 10% added inactivated fetal cow serum.

Cells used:

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ATDC5: Sub-strain derived from mouse teratocarcinoma AT805 cells; cartilage and chromatophore specific; alkaliphosphatase positive

HEK293: Human fetal kidney-derived cells

NIH3T3: Mouse fetus-derived fibroblasts

5   Transfection and reporter assay were performed as follows.

The cells were prepared 2 x 10^4/well on 24-well plates. 24 hours later, they were transfected (Biochem.Roche) using a FUGENE 6 kit in accordance with the attached manual. 50 ng each of CMV-β-gal was used to correct the transfection efficiency, and the total vector alone was added to a total of 150 ng.

10   The protein was collected 30 hours after transfection. The medium was discarded, and after washing with PBS the cell lysate was collected using 100 μl of Passive dissolution buffer (Promega). Next, 20 μl of this cell lysate was transferred to a 96-well plate, and luciferase activity was measured with a luminometer. β-gal activity was also measured with a plate reader, and the Luc value was then corrected by dividing with the β-gal value. β-gal

15   staining was done as follows. Using X-gal as the β-galactosidase, stained (5-bromo-4-chloro-3-indolyl- β- D-galactopyranoside; Sigma). Embryos were fixed for 20 minutes in 4% paraformaldehyde, soaked in X-gal solution, and stained for 12 to 24 hours at 37°C. After staining they were washed in PBS, and fixed again in 4% paraformaldehyde.

As a result, transcription activity was reduced by from 10 to 30% by deleting the

20   region (12 nucleotides) from -84 to -73 bp of the promoter sequence (Figures 1a, 1b).

The region from −1 to −114 including the aforementioned deleted region is 94% homologous between mice and humans, while the 12 nucleotides are 100% homologous. Analysis using a computer with Bioinformatix analysis software (http://www.cbrc.jp/ research /db/TFSEARCHJ.html) showed the presence of the EBS (Ets binding site) in these 12

25   nucleotides (Figure 1c).

Next, the inventors introduced mutations into the transcriptional factor binding sequences of the previous and subsequent regions including that region, and investigated the effects on transcription of those sites using various cell lines.

As shown in Figure 1d, activity was reduced by from about 9 to 40% in almost all cell

30   lines by point mutations in the EBS (also called the GBS, shown as "GBS" in Figure 1d). This suggests that this EBS is an essential element for constitutive expression of synoviolin.

[Example 3]   Identification of transcriptional factors for EBS (from -85 to -70)

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The Ets family is formed of at least 30 members all of which have the Ets domain, which is a DNA binding domain. Its central sequence is GGAA, and the sequence following that sequence is thought to be important for binding to the sequence.

First, probes comprising the 12-nucleotide sequence were used to investigate whether or not transcriptional factors actually bind to that sequence. Transcription factor binding was tested by gel shift assay (EMSA).

EMSA was performed as follows.

Probes:

EBS WT (–85 to –70): GCGCCGCCGGAAGTGA (SEQ ID: NO 6)

EBS MT (–85 to –70): GCGCCGCCGTAAGTGA (SEQ ID: NO 7)

The EMSA probes were prepared by annealing sense chains and antisense chains with 25-nucleotide sequences for 10 minutes at 90°C.

Next, T4 nuclease kinase, buffer and $\gamma^{32}P$ were mixed and the mixture was reacted for 30 minutes at room temperature. The reaction solution was passed through a Micro Spin G-25 column (Amersham Pharmacia Biotech), and the collected column fraction was centrifuged to obtain labeled probes. Those having radioactivity of 30000 cpm or more per 1 μl were used.

10 μg of protein was mixed with reaction buffer and probe, and reacted for 30 minutes at room temperature. For purposes of supershift, they were reacted for 1 hour at 4°C before being reacted with the probe. The reaction solution was electrophoresed for about 3 hours at 100v, 50 mA in non-denatured gel. The gel was dried, and analyzed by autoradiography using a Fujii BAS2000.

As a result, 4 bands (bands at locations a, b, c and d in Figure 2a) were formed by gel shift using NIH3T3 nuclear extract. In cold competition (competitive testing using unlabeled probe), all bands disappeared, and no interference was seen with a probe having a single-nucleotide mutation (Figure 2a).

When cold competition was attempted using Ets1/Pea3 and Ets probes to see what Ets family members would bind to the promoter sequence, competitive interference occurred with the ets1/pea3 probe, and interference was eliminated by mutation (lanes 5 and 6 in Figure 2b).

This suggests that the factors that bind to the promoter sequence may be the Ets 1, Pea 3, GABPα and other transcriptional factors which are factors which bind to the Ets1/Pea3 probe (purchased from Santa Cruz Co.). In supershift testing using a variety of antibodies,

GABPα and Tel supershifted while Fli-1 exhibited an interference effect (lanes 5 and 6 in Figure 2b, lanes 6, 7 and 8 and 10, 11 and 12 in Figure 2c). When a gel shift test was performed using the in vitro translation products of GABPα, Fli-1 and ets1, all exhibited a supershift. This indicates that multiple members of the Ets family bind to this sequence.

Next, complex formation with GABPβ was tested using supershifted GABPα. In gel shift testing using the respective in vitro translation products, a new band a,b (complex) was formed in lane 7, in which the GABPβ protein was added to the GABPα protein. Addition of GABPβ antibodies interfered with the formation of complex a (lanes 10 and 11 in Figure 2d).

These results show that GABPα/β form a complex on the EBS of the synoviolin promoter (Figure 2d).

[Example 4]   Transcriptional regulation by the Ets family in NIH3T3 and effects of GABPα, Fli-1 and Ets1 on synoviolin transcription activity

The synoviolin transcriptional activation ability in cells of transcriptional factors which bind to the EBS was evaluated by a transcriptional activation assay.

(1)   Oligo preparation of ODNs and preparation of cell extract

20-nucleotide oligodeoxyribonucleotides (ODNs) were obtained by chemical synthesis. A decoy ODN was also prepared by annealing of sense and antisense oligonucleotides. Cells in the logarithmic growth phase were treated with trypsin and transferred to 96-well plates (1×10^3/well). After 24 hours, 20 pmol of decoy ODN was added to each well, and transfection was performed for 3 days using LipofectAMINE 2000 (Invitrogen, San Diego, CA) in accordance with the instructions. A cell proliferation assay was performed using Alamar Blue (Biosource International) in accordance with the kit's instructions to determine proliferation of cells having the decoy ODN.

(2)   Oligo preparation of RNAi and preparation of cell extract

21-nucleotide RNA was obtained by chemical synthesis. siRNA was prepared in accordance with the protocols of Elbashir et al (Elbashir, S.M., *Nature* 411: 494-498, 2001). Cells in the logarithmic growth phase were treated with trypsin and transferred to 96-well plates (1×10^3/well). After 24 hours, 20 pmol of siRNA was added to each well, and transfection was performed for 3 days using LipofectAMINE 2000 (Invitrogen, San Diego, CA) in accordance with the instructions. A cell proliferation assay was performed using Alamar Blue (Biosource International) in accordance with the kit's instructions to determine proliferation of the siRNA cells.

(3)   Results

In NIH3T3, Fli-1 suppressed synoviolin transcription activity, while GABPα increased transcription activity (Figures 3a, 3b). Synoviolin transcription was also lower in NIH3T3 cells in which the GABPα and GABPβ RNAi was knocked out (Figure 3c). This suggests that GABPα/β is responsible for expression of synoviolin in NIH3T3 cells.

Next, to show that this GABPα/β complex is responsible for transcription activity of synoviolin via the EBS, a transcription activity assay was performed using promoters (−200 to +843) having an EBS (G-76T) mutant and the wild EBS, respectively.

As a result, transcriptional activation with the wild type was about three times that with the mutant even though the mutant was not activated at all.

(4)   Western blotting

Next, NIH3T3 cells were treated with 200 nM of decoy, and synoviolin expression was evaluated by western blotting.

Western blotting analysis was performed as follows. Cell culture was collected, and dissolved in a solution comprising 1% NP-40, 25 mM Tris-HCl, pH 6.8, 0.25% SDS, 0.05% 2-mercaptoethanol and 0.1% glycerol. An aliquot of the clear cell lysate was isolated on SDS-polyacrylamide gel. The isolated protein was transferred to a nitrocellulose membrane, and immunoblotted using anti-synoviolin monoclonal antibodies. The bound antibodies were detected with peroxidase-binding goat anti-mouse immunoglobulin and an ECL detection system (Amersham Pharmacia Biotech).

As a result, expression of synoviolin was reduced by roughly half by treatment with the EBS wild type.

These data suggest that synoviolin transcription is regulated by GABPα/β via the EBS.

[Example 5]   Identification of sites necessary for synoviolin expression in mouse embryos

Next, a transgenic mouse in which a plasmid which the synoviolin promoter bound to LacZ was overexpressed was prepared to confirm the effects of EBS in vivo in mouse embryos. 4 Tgs were prepared having full-length 3k and 1k promoters and such promoters with single-nucleotide mutations, respectively (Figure 4a).

The transgenic (Tg) mouse constructions and Tg mice were prepared as follows.

Roughly 3k fragments were removed from SyG-2.2k-BV2 with NotI and NcoI, and inserted into SyTB/pbs to prepared SyL-2.2kwt/pbs. Similarly, SyL-2.2kmG-76T/pbs, SyL-200wt/pbs and SyL-200mG-76T/pbs were prepared using fragments extracted from SyG. The

constructs were each purified using a QIAGEN Plasmid Kit (QIAGEN), and DNA linearized with ScaI was directly inserted by microporation into the nuclei of fertilized eggs of BDF mice (offspring from cross-breeding of C57BL/6N and DBA/2N), and transferred to the uterine tubes of surrogate mothers. Genomes were extracted from the tails of mice born from 8 to 9 uteri, which were confirmed from southern blotting to be Tg mice.

Embryos were X-gal stained to test synoviolin expression from these Tg mice. Expression of LacZ in knockout mouse embryos with LacZ knocked in was compared with expression in the 4 Tg mice prepared here.

11.5 and 14.5 d.p.c. (days post coitus) synoviolin embryos were stained with LacZ. The transgenic mice having introduced 3k and 1k promoters exhibited staining in roughly the same locations as the hetero-knockout mice. Surprisingly, it was shown that in the transgenic mice having introduced 3k and 1k promoters with introduced single-nucleotide mutations, the expression sites were randomized (Figure 4b).

Similar results have been reported when a region necessary for transcriptional activation is eliminated (pax5, col11a2, etc.). This shows that EBS is a site necessary for transcription of synoviolin.

When this expression was compared to GABPα expression using a 13.5 d.p.c. embryo, expression was seen in roughly the same sites (Figure 4c). These results suggest that GABPα regulates synoviolin via the EBS in embryogenesis as well as in vitro.

[Example 6]   Expression of synoviolin in RA synovial cells

When the effects of GABPα via EBS in rheumatic synovial cells were studied, a supershift was found with GABPα as it was with the NIH3T3 extracts in a gel shift using nuclear extracts of synovial cells. Regulation of synoviolin expression was also investigated using an EBS decoy to test the significance of EBS in rheumatic synovial cells or in other words to confirm that transcriptional regulation by GABPα via EBS leads in turn to regulation of synoviolin expression and ultimately to regulation of synovial cell proliferation.

As a result, expression was reduced by roughly half (Figure 5).

These results show that GABPα/β is responsible for constitutive gene expression of synoviolin in RA synovial cells.

[Example 7]   Annexin V staining and FACS analysis

NIH3T3 cells were treated with trypsin, washed twice with chilled PBS, suspended in

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1 x annexin-binding buffer (Vybrant apoptosis assay kit, Invitrogen), and prepared to a concentration of 1 x 10^6 cells/ml. 100 µl of cell suspension was used per test. 5 µg of FITC-labeled Annexin V and 1 µl of PI working solution were added and incubated for 15 minutes at room temperature, 400 µl of 1 x annexin-binding buffer was added and gently stirred, and

5   this was analyzed with FACSCalibur (Becton Dickinson).

Since synoviolin is resistant to apoptosis induced by ER stress, the following experiment was performed to clarify the relation between synoviolin levels and apoptosis.

NIH3T3 cells were prepared and after 24 hours siRNA (25 nM) of GFP or synoviolin was transfected into the NIH3T3 cells using Lipofectamine 2000 (Invitrogen), and incubated

10   for 84 hours. An EBS decoy ODN was prepared as above and transfected into the NIH3T3 cells.

As a result, apoptosis was induced when the level of synoviolin expression was depressed in NIH3T3 cells using synoviolin RNAi (Figure 6a). In Figure 6a, the upper panel shows the results of western blotting, while the lower panel shows photomicrographs of

15   NIH3T3 cells (100x). Similar, when the effect of transcriptional inhibition of synoviolin using EBS decoy nucleic acid was investigated, apoptosis was induced in NIH3T3 cells (Figure 6b). In Figure 6b, the upper panel shows the results of western blotting, while the lower panel shows photomicrographs of NIH3T3 cells (100x).

NIH3T3 cells overexpressing synoviolin were also prepared (Figure 7a) and used to

20   investigate the effects of EBS decoy nucleic acid.

To establish a stable cell line overexpressing synoviolin, NIH3T3 cells were transfected with Lipofectamine 2000 (Invitrogen), and 24 hours later were diluted 10 times with fresh growth medium and subcultured. The next day, selection medium (containing 0.5 µg/ml G418) was added, and a clone stably expressing a HA-Synoviolin-HAHA/pcDNA3

25   expression vector was obtained. A HA-pcDNA3 empty vector was used as a control. To confirm expression from the plasmid in each cell line, western blotting was performed using antibodies to the HA tag.

Apoptosis induction with EBS decoy ODNs was tested as follows.

84 hours after transfection of EBS with FUGENE6 (Roche) reagent, the cells were

30   collected, placed in a microtube and labeled with Annexin V-FITC. The distribution of live cells and cells which had undergone apoptosis was analyzed be FACS analysis. Western blotting was also performed using synoviolin antibodies. Beta-actin antibodies were used as a control, and HA antibodies were used to confirm stable expression.

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The results are shown in Figure 7. Cells overexpressing synoviolin were resistant to apoptosis caused by EBS decoy nucleic acid (Figure 7b, Figure 7c). Moreover, in an assay of apoptosis by FACS using Annexin V, the apoptosis rate of the normal NIH3T3 cells was 51.1% while that of the cells overexpressing synoviolin was only 29.8% (Figure 7d). Figure 7c is a photomicrograph of NIH3T3 cells at a magnification of 100.

These results show that when transcriptional regulation via EBS is suppressed, synoviolin expression is also suppressed and apoptosis is induced in NIH3T3 cells.

INDUSTRIAL APPLICABILITY

A synoviolin promoter is provided by the present invention. The promoter of the present invention is useful because it regulates synoviolin expression, and also can be used for treating various disorders (such as rheumatism) by causing expression of useful genes at sites of synoviolin expression.

Sequence Listing Free Text

SEQ ID: NO 3:	Synthetic DNA
SEQ ID: NO 4:	Synthetic DNA
SEQ ID: NO 5:	Synthetic DNA
SEQ ID: NO 6:	Synthetic DNA
SEQ ID: NO 7:	Synthetic DNA